Published in final edited form as:

J Great Lakes Res. 2014 December 1; 40(4): 879–885. doi:10.1016/j.jglr.2014.08.004.

***In vivo* and *in vitro* phenotypic differences between Great Lakes VHSV genotype IVb isolates with sequence types vcG001 and vcG002**

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Abstract

Viral hemorrhagic septicemia virus (VHSV) is an aquatic rhabdovirus first recognized in farmed rainbow trout in Denmark. In the past decade, a new genotype of this virus, IVb was discovered in the Laurentian Great Lakes basin and has caused several massive die-offs in some of the 28 species of susceptible North American freshwater fishes. Since its colonization of the Great Lakes, several closely related sequence types within genotype IVb have been reported, the two most common of which are vcG001 and vcG002. These sequence types have different spatial distributions in the Great Lakes. The aim of this study was to determine whether the genotypic differences between representative vcG001 (isolate MI03) and vcG002 (isolate 2010-030 #91) isolates correspond to phenotypic differences in terms of virulence using both an *in vitro* and *in vivo* approach. *In vitro* infection of epithelioma papulosum cyprini (EPC), bluegill fry (BF-2), and Chinook salmon embryo (CHSE) cells demonstrated some differences in onset and rate of growth in EPC and BF-2 cells, without any difference in the quantity of RNA produced. *In vivo* infection of round gobies (*Neogobius melanostomus*) via immersion exposure to different concentrations of vcG001 or vcG002 caused a significantly greater mortality in round gobies exposed to 10² plaque forming units ml⁻¹ of vcG001. These experiments suggest that there are phenotypic differences between Great Lakes isolates of VHSV genotype IVb.

Keywords

genetic diversity; *Neogobius melanostomus*; round goby; sequence type; viral hemorrhagic septicemia virus

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Introduction

Viral hemorrhagic septicemia virus (VHSV) is an enveloped, negative-sense, ssRNA virus (Genus: *Novirhabdovirus*, Family: *Rhabdoviridae*) that was first reported in 1938 in farmed Danish rainbow trout (Schäperclaus, 1938). Extensively studied in Europe, VHSV was not thought to be a pathogen of concern in North America until 1988, when it was detected in spawning salmon in Washington, USA (Brunson et al., 1989). In 2005, VHSV was detected in the Great Lakes, with subsequent testing showing its presence as early as 2003 (Elsayed et al., 2006). By 2009, the virus had been detected in all five Great Lakes (Cornwell et al., 2011). In phylogenetic analyses the Great Lakes genotype, denoted VHSV IVb, is most closely related to marine isolates from the Atlantic Coast of North America (Elsayed et al., 2006; Thompson et al., 2011), suggesting that the virus colonized the Great Lakes from neighboring marine environments. RNA viruses tend to have intrinsically high mutation rates (Santiago and Sanjuan, 2005), and VHSV has shown particularly high adaptability in new geographic ranges and host species, a characteristic that makes it one of only nine World Organization for Animal Health (OIE) reportable fish diseases (OIE, 2011).

Currently, 28 different species of freshwater fishes in the Great Lakes basin are known to be susceptible to VHSV and are regulated by the VHSV Federal Order in the United States (USDA, 2008). The list of regulated species includes economically and recreationally important fish. For this reason, VHSV IVb is viewed as a significant threat to the Great Lakes' freshwater fisheries, as well as the tourism, aquaculture, and baitfish industries (Bain et al., 2010; Faisal et al., 2012). VHSV-susceptible fish species have been grouped as either highly susceptible (e.g. muskellunge *E. masquinongy*) (Kim and Faisal, 2010), moderately susceptible (e.g. walleye *Sander vitreus*) (Groocock et al., 2012), or resistant (e.g. rainbow trout *Oncorhynchus mykiss*) (Al-Hussinee et al., 2010). The round goby (*Neogobius melanostomus*), an invasive species of the Great Lakes originally from the Ponto-Caspian region, is particularly susceptible to VHSV and has experienced mass mortality events attributed to the virus (Groocock et al., 2007). Additionally, the round goby is potentially more responsible for the maintenance and spread of VHSV than others, and may be a better species to target for VHSV surveillance (Cornwell et al., 2012b). In a recent study by Cornwell et al. (2012a), the round goby was shown to carry higher median viral loads than yellow perch (*Perca flavescens*), implying that round gobies may be able to replicate greater levels of VHSV before showing clinical signs of disease.

VHSV is horizontally transmissible with attachment at the gills or skin of a new host (Chilmonczyk et al., 1995; Harmache et al., 2006). The ability of an isolate to attach to the host has been shown to affect virulence (Brudseth et al., 2008); however the exact mechanism of external entry to the host is still unclear. Internally, VHSV targets the endothelial lining of blood vessels (Faisal et al., 2012), resulting in hemorrhages in locations such as the dermal, periocular, peritoneal, and visceral regions as well as the fin bases (Cornwell et al., 2012b). Other lesions associated with the virus include darkening of the skin, anemia resulting in gill and hepatic pallor, exophthalmia, and serous or serosanguineous abdominal ascites (Al-Hussinee et al., 2011; Cornwell et al., 2012b; Yasutake, 1975). VHSV affects fish in an acute, subacute, or chronic manner (Wolf, 1988).

RNA sequences from the *glycoprotein (G)* gene of VHSV have been useful tools for sequence type identification (Thompson et al., 2011). The *G* gene encodes for a surface antigen that aids in viral entry into the cell membrane. The sequence of the central *G* region, consisting of 669 nucleotides in the middle of the 1609-nucleotide *G* gene, has been used to distinguish 11 different sequence types of VHSV IVb as of 2009; isolates with identical central *G* region sequences are classified into the same sequence type (Thompson et al., 2011). These different sequence types did not correlate with host species or year of isolation, but there was some spatial heterogeneity in the distribution of the two most common types, with vcG001 present throughout most of the Great Lakes basin and vcG002 more commonly detected in the southeastern waters of Lake Ontario and the St. Lawrence Seaway (Thompson et al., 2011). Within the Great Lakes, VHSV has a very low but rising sequence diversity based on the central *G* region (Cornwell et al., 2012a, Thompson et al., 2011).

Although there is evidence for limited genotypic diversity within genotype IVb, there has been no demonstration of phenotypic variation among virus isolates within genotype IVb. The aim of this study was to examine two virus isolates selected to represent sequence types vcG001 and vcG002 and determine if they differ in virulence *in vitro* in three different cell lines and *in vivo* using a round goby model.

Material and Methods

Virus stocks

The MI03 isolate (Elsayed et al., 2006, GenBank Accession No. GQ385941.1) is considered the index isolate of VHSV IVb and was used as the vcG001 sequence type representative; the source stock obtained for this study had been passaged twice in epithelioma papulosum cyprini (EPC) cells prior to use (Winton et al., 2010). An isolate from round goby (2010-030 #91) collected in the St. Lawrence River, NY, was used as a representative for the vcG002 sequence type (GenBank Accession No. EF564588.1) and was also passaged twice in EPC cells prior to use. For both sequence types, a concentrated stock virus was prepared in EPC cells using a 0.005 multiplicity of infection (MOI), with a resulting titer determined by plaque assay (Batts and Winton, 2007). Prepared viral stocks were stored in 1 mL aliquots at -80°C . The same stock preparation was used in all experiments. Sequence type was re-confirmed after preparation of the stock through sequencing of the central *G* region (Thompson et al., 2011).

In vitro Phenotypic Characteristics

In vitro growth curves were performed in three fish cell lines: EPC, bluegill fry (BF-2), and Chinook salmon embryo (CHSE). Three separate growth curves were conducted for each cell line, with three replicates per growth curve. For each replicate, 10 wells of a 24-well plate were inoculated at 0.005 MOI, based on plaque forming units of the virus stocks. At each time point (0, 5-6, 12, 24, 48, 72, 96, 120, 133, or 168 h post inoculation), the cells and supernatant from one well from each replicate were harvested and used in a plaque assay to determine titer (Groocock et al., 2012). The remaining fluid (including both cells and supernatant) from that time point was frozen at -80°C for later RNA extraction and quantification of viral RNA by qRT-PCR.

In-vivo Phenotypic Characteristics

Round gobies were obtained at Fort Niagara State Park in Youngstown, New York (43° 15' 39.6" N, 79° 3' 34.35" W) by seining under a New York Department of Environmental Conservation scientific collection permit. All fish (n=540) were transported to Cornell University immediately upon collection, where they were divided into two 700 L Living Stream tanks (Frigid Units, Toledo, Ohio) with a flow rate of 0.6 L min⁻¹. Round gobies were fed daily on frozen bloodworms at approximately 1% body weight (based on average weight of a sample of 10 fish), and the temperature was monitored once daily and remained at 10 ± 1 °C. Effluent from all tanks was disinfected through hyperchlorination with 12.5% sodium hypochlorite to maintain a free residual chlorine concentration of at least 1.0 mg L⁻¹ for at least 10 min prior to discharge into the city water treatment plant. The use of all animals in this study was approved by the Cornell University Institutional Animal Care and Use Committee.

Wild caught round gobies were used in this experiment because no disease free, cultured source of round gobies were available and we were interested in testing differences in this species in particular because of their high susceptibility to VHSV IVb, the observation that a high proportion of them are infected in the field (Cornwell et al. 2012a), and their potential ability to spread VHSV (Cornwell et al. 2012b). To reduce the possibility that any collected fish had been previously exposed to VHSV, we collected fish from a site where we have never detected VHSV, despite 5 consecutive years of surveillance. Use of serology would have been helpful in confirming the naïve disease history of these fish, however, they were too small to allow non-lethal blood collection in quantities sufficient for serology. Therefore, we relied on the randomized nature of the experimental design (described below) as well as experimental replicates to account for differences in disease exposure history among round gobies used in this experiment.

In vivo virulence was assessed in duplicate using a 24 h immersion exposure of round gobies to three different concentrations of the two sequence types at 13 °C. For exposure to each sequence type, fish (n=240) were randomly allocated into six 3 L tanks (40 fish per tank) using an online list randomizer (www.random.org; Haahr and Haahr, 1998; Fig. 1) and exposed to one of 3 doses of VHSV (10² plaque forming units [pfu] ml⁻¹, 10³ pfu mL⁻¹, or 10⁴ pfu mL⁻¹) of vcG001 or vcG002. Controls (n=20) were exposed to the same volume of the cell culture media used to dilute the virus. To monitor viral concentration in individual fish over time, fish in each replicate tank were individually marked with a fluorescent elastomer tag (Northwest Marine Technology, Shaw Island, Washington) by subdermal injection one of five colors on either the right or left of the dorsal fin.

After the 24 h exposure period, fish from each tank were randomly allocated into two 9 L duplicate tanks of 20 fish (10 for controls; Fig. 1), using the same method for randomization; this was considered time zero for the *in vivo* experiments. Replicate tanks of each sequence type were placed in a random order on concrete blocks within two flow-through Living Streams. Water entered each tank via separate tubing from a PVC manifold and exited through the side of the tank into the Living Stream. The round gobies were checked twice a day for clinical signs, lesions, and mortalities.

Fin clips were taken from all gobies once a week (Cornwell et al., 2013) and stored in RNALater (Life Technologies, Carlsbad, California) at -80°C for RNA extraction and viral RNA quantification by qRT-PCR. Dead round gobies were either necropsied when found or frozen at -80°C until a necropsy could be performed. RNA was extracted for qRT-PCR from both a fin clip and a pooled internal organ (liver, kidney, spleen, heart, and brain) sample from all fish that died during the experiment and gross internal and external lesions were documented along with the sex and identity of the fish. Fin clips from necropsied fish were treated identical to the weekly fin clips described above; the pooled organs were weighed and diluted 1/10 (w/v) in HMEM-10% (HMEM with 10% fetal bovine serum). A 1.3 mm chrome steel bead (Bio-Spec Products, Dover, Florida) was added to the pooled organs and tissues were homogenized using a Bead Beater (Bio-Spec Products) for 1 min then centrifuged at $8000 \times g$ for 1 min. Pooled organ homogenate was immediately plated on cells for viral isolation (see below), and the remaining homogenate stored at -80°C for RNA extraction.

RNA extraction and Quantitative RT- PCR

To prepare for RNA extraction, 200 μL of HMEM-10% and a 1.3 mm steel chrome bead (Bio-Spec Products) were added to the fin samples, which were then homogenized using a Bead beater (Bio-Spec Products) for 1 min and centrifuged at $8000 \times g$ for 1 min. RNA extraction was performed for all samples using a MagMax magnetic bead extraction system and MagMax-96 viral RNA isolation kit (Life Technologies, Carlsbad, California) using the protocols described in the kit and extraction program AM1836_DW_50_V2 (Cornwell et al., 2012b). Sample RNA quantity and quality was assessed using a NanoVue Plus spectrophotometer (GE Healthcare, Piscataway, New Jersey).

Quantification of viral RNA was performed using the qRT-PCR assay described by Hope et al. (2010), with modifications described in Cornwell et al. (2012a). Each sample was run in duplicate on a Applied Biosystems PRISM® model 7500 real-time PCR system (Life Technologies). A standard regression fit using software from the supplier was used to determine copy numbers in unknown samples.

Tissue collection and virus isolation in cell culture

A portion of the pooled organ homogenate from fish that died during the trial was further diluted to 1/50 and filtered through a 0.45 μm sterile Acrodisc syringe filter (Pall Corporation, Port Washington, New York). A portion of the filtered 1/50 homogenate was further diluted to 1/250 and both the 1/50 and 1/250 dilutions were plated in triplicate onto 48-well plates containing a confluent monolayer of EPC cells in HMEM-5% media for a final in-well dilution of 1/100 and 1/500, respectively. Plates were incubated at 15°C for two weeks, after which each well was passaged to a new 48-well plate containing a confluent monolayer of EPC cells and monitored for CPE (cytopathic effect) once weekly for at least two weeks. Presence of CPE after the second passage was assumed to be due to the replication of VHSV.

Statistical Analysis

For the *in vitro* experiment, two-tailed t-tests were used to test for differences in slope between the two sequence types in each cell line (JMP 9.0.2, SAS Institute, Cary, North Carolina). For the *in vivo* experiment, Chi-square tests were used to test differences in mortality, viral load between fin clip samples and pooled organ samples, frequency of clinical signs, length of dead vs. survived fish, and the mean viral load over time between the two sequence types.

Results

In Vitro Phenotypic Characteristics

In CHSE cells, the exponential phase of infectious viral particle production occurred between 12 and 72 h for both sequence types, although the peak virus production was not reached until 120 h (Fig. 2a). There was no significant difference in the slope of the exponential phase between vcG001 (1.17 ± 0.023 ; mean $\pm$ SD) and vcG002 (1.17 ± 0.007) in CHSE cells and there was no difference in the final amount of infectious particles produced in this cell line ($t = -1.7$, $p = 0.091$). The slope of the exponential phase for viral RNA production in CHSE cells also occurred between 12 and 72 h for both sequence types (Fig. 2d), and there was no significant difference in the slope of the exponential phase between the two sequence types ($t = -2.54$, $p = 0.056$). Similarly, there was no difference in the final amount of viral RNA (Fig. 2d) produced in the plateau phase between the two sequence types in CHSE cells ($t = 0.94$, $p = 0.35$).

In BF-2 cells, the exponential phase of infectious viral particle production occurred between 12 and 48 h for both sequence types (Fig. 2b). There was a significant difference ($t = 11.58$, $p < 0.001$) in the slopes of the exponential phase between the two sequence types. Although vcG001 initially replicated at a faster rate (between 6 and 12 h), overall during the exponential phase vcG002 replicated at a faster rate (1.28 ± 0.0017) than vcG001 (1.24 ± 0.0074). However, vcG001 overall replicated to a significantly higher titer than vcG002 ($t = -7.027$, $p < 0.001$). The slope of the exponential phase for RNA production in BF-2 cells occurred between 12 and 48 h for both sequence types and there was no significant difference in the slope of the exponential phase between the two sequence types ($t = -2.88$, $p = 0.056$). The amount of total viral RNA produced in BF-2 cells in three different experiments with three replicates each was also not significantly different between the two sequence types ($t = -0.088$, $p = 0.93$) (Fig. 2e).

In EPC cells, the exponential phase of infectious viral particle production occurred between 12 and 48 h for vcG001 and between 24 and 72 h for vcG002 (Fig. 2c). The slope of the exponential phase for vcG002 was significantly shallower than for vcG001 in EPC cells ($t = -29.59$; $p < 0.0001$). The mean slope during the exponential phase of growth for vcG001 inoculated onto EPC cells was 1.27 ± 0.0063 , while the mean slope of the exponential phase of vcG002 inoculated onto EPC cells was 1.13 ± 0.0067 . The lag phase of vcG002 extended 6 hours beyond the lag phase for vcG001 for infectious particle production. The plateau phase for infectious viral particle production in EPC cells was reached at around 72 h post-infection for vcG001, and 96 h for vcG002. The plaque assay results showed that vcG001

had a significantly larger ($t=-47.84$, $p<0.0001$) number of infectious viral particles than vcG002 in EPC cells across three experiments with three replicates each. For viral RNA production, the amount of RNA present at 0 h was approximately one log higher in vcG002 than in vcG001, however vcG001 surpassed vcG002 by 24 h. The lag phase of vcG002 extended 6 h beyond the lag phase for vcG001 for viral RNA. The exponential phase for RNA production for vcG001 in EPC cells occurred between time points 6 and 24 h and was significantly greater ($t = 11.128$; $p < 0.0001$) than that of vcG002 in EPC cells, which occurred between time points 12 and 48 h. However, the qRT-PCR results showed no significant difference ($t=-1.09753$, $p=0.2812$) between the viral N gene copy numbers of the two sequence types during the plateau phase in EPC cells (Fig. 2f).

Across all three cell types, there was a significant difference in the final titer of virus produced (vcG001: $F = 592.05$; $p < 0.0001$; vcG002: $F = 192.36$, $p < 0.0001$). For both sequence types, inoculation of CHSE cells resulted in the lowest infectious titer. For vcG001, inoculation in EPC cells resulted in the highest titers while for vcG002, inoculation in BF-2 cells resulted in the highest titer. There was also a significant difference in the amount of viral RNA eventually produced across all three cell types (vcG001: $F = 160.86$, $p < 0.001$; vcG002: $F = 147.50$, $p < 0.001$). For both sequence types, the highest amount of viral RNA was produced when virus was inoculated in EPC cells.

In Vivo Phenotypic Characteristics

A total of 71 fish died during this experiment with a mean length of 59.87 mm (range 40-88 mm), a mean weight of 2.72 g (range 0.6-7.4 g), and a male:female ratio of 8:9 (survivors were not sexed). Quantitative RT-PCR and cell culture confirmed that 70 out of 71 fish tested positive for VHSV. No fish in the control tanks died during the experiment, and all qRT-PCR results on fin clips from control fish were negative. The first fish testing positive for VHSV by qRT-PCR was found dead on day 4 of the infection trial and had been challenged via immersion with a 10^4 pfu mL^{-1} concentration of vcG001 (Fig. 3c). The average survivorship over time was significantly less ($\chi^2 = 19.13$, $p < 0.0001$, d.f.=1) for the 10^2 dosage group of vcG001 than for vcG002 (Fig. 3a). However, average survivorship was not significantly different for the 10^3 ($\chi^2 = 1.9$; $p = 0.17$, d.f.=1) and 10^4 ($\chi^2 = 0.48$, $p = 0.49$, d.f.=1) dosage groups (Fig. 3b; 3c). There was a clear dose response for vcG002, with average cumulative mortality increasing from 2% at 10^2 to 22% at 10^3 and reaching 41% by 10^4 pfu mL^{-1} (Fig. 3). This dose response was not observed with vcG001.

Lesions noted at time of fish necropsy were compared between dosages and sequence types. Typical signs found included hemorrhaging on fins, petechial hemorrhaging on the body, cranial hemorrhaging, serosanguinous ascites, hemorrhaging of internal organs such as the liver, hemorrhaging on vent, and dermal pallor. There was no significant difference between the prevalence of any of these clinical signs between the two sequence types (Table 1). The most common symptom exhibited by VHSV type IVb infection was hemorrhaging in the fins, particularly the pectoral, caudal and pelvic fins (Fig. 4).

At necropsy, there were no significant differences in the viral loads (VHSV N gene copies per 50 ng RNA) between samples taken from a fin clip and from pooled internal organs in both vcG001 and vcG002 when all doses were combined. Although there was not a strong

correlation between the quantity of viral RNA in the pooled organ sample and in the fin clip sample ($r^2 = 0.25$), the qualitative agreement between the two was very high; only 1 out of 70 fish tested positive for the pool and negative for the fin clip. There was no significant difference between the viral load over time of fish infected with vcG001 or vcG002 (Fig. 5a). Similarly, there was no significant difference between the immersion dosages of virus, nor the sequence type with regard to viral load fish that died during the trial or in fish that were necropsied at the end of the trial (Fig. 5b).

Discussion

Viral hemorrhagic septicemia virus genotype IVb is a pathogen in the Laurentian Great Lakes Basin, and has caused several mortality events affecting ecologically and recreationally important fish. Although 11 different sequence types have been classified from this VHSV genotype, how the sequence types differ phenotypically in their virulence has not been studied (Thompson et al., 2011). There is a known synonymous mutation in the glycoprotein gene between vcG001 and vcG002, the two most commonly identified sequence types of genotype IVb (Thompson et al., 2011). Although synonymous mutations can result in phenotypic changes that affect viral fitness, especially in RNA viruses (Cuevas et al., 2012), it is also possible that one or more additional mutations exist between these two sequence types that may account for the differences observed. The full length sequence for vcG001 has been published (Ammayappan et al., 2011); but in the future, it will be useful to obtain full length sequences for other sequence types, especially vcG002, to further investigate the causes behind these differences in virulence. The experiments presented in this manuscript show that although isolates from sequence types vcG001 and vcG002 have similar characteristics in general, there are also significant differences in *in vitro* production of viable infectious particles as well as some difference in virulence *in vivo*.

In Vitro Phenotypic Characteristics

There were significant differences in the *in vitro* phenotypic characteristics between vcG001 and vcG002 and these differences varied across cell types. In CHSE cells, there was no difference between either the slope of the exponential phase of replication or the final amount of infectious viral particles produced (Fig. 2a). In BF-2 cells, vcG002 replicated at a significantly faster rate overall during the exponential phase than vcG001, but vcG001 still reached a significantly higher titer at most time points than vcG002 (Fig. 2b). In contrast, in EPC cells, vcG001 replicated at a significantly higher rate and the exponential phase began sooner than vcG002. In EPC cells, vcG001 still reached a significantly higher titer than vcG002, as seen in the BF-2 cells (Fig. 2c). With three separate growth curves run in triplicate for each cell line, these results suggest that the phenotypic characteristics of sequence types of VHSV genotype IVb are not constant across cell types. They also suggest that different sequence types may grow better on different cell types.

The two sequence types produced similar amounts of viral RNA in each cell line (Fig. 2 d-f). vcG001 inoculated in EPC cells had a significantly steeper slope of viral RNA production than vcG002; no significant difference in slope was seen between the two sequence types in BF-2 or CHSE cells. This discordance between pfu and viral RNA was observed in the stock

virus as well, with the vcG001 stock having a pfu:viral RNA ratio of 22.9 and vcG002 of 0.5. These results imply that the discordance between the viral RNA and infectious viral particle results may be due to the inefficiency of vcG002 in forming infectious virus particles, not its ability to produce viral RNA. Additionally, because the qRT-PCR primers and probes used in this study quantify both viral genomic RNA and viral mRNA, the discordances observed between infectious viral particles and viral RNA levels could be due to differences in the viral replication cycle between the two sequence types. Alternatively, it is possible that vcG002 has a higher proportion of defective interfering particles in their population than vcG001, which may account for the difference in virulence (Huang, 1973; Holland et al., 1976). Host factors may also contribute, including epigenetic differences involving in the enveloping of VHSV particles, which could decouple viral load from virulence.

In Vivo Phenotypic Characteristics

The immersion infection of 120 round gobies with the three different dosages of either vcG001 or vcG002 showed a difference in virulence between the sequence types. There was a significant difference between the mortality of the two sequence types at the 10^2 pfu mL⁻¹ dosages, but not for the 10^3 and 10^4 pfu mL⁻¹ dosages. It is possible that, once round gobies were exposed to a high enough dose, the host was overwhelmed and differences attributable to variation in sequence type were masked. The *in vivo* experiments reported here were conducted in duplicate with each treatment challenged as a single group of 40 fish and then split into two groups of 20 fish each to provide internal replication for the holding conditions. In the future, additional experiments to repeat this work and test additional VHSV isolates from sequence types vcG001 and vcG002 will be needed to determine the reproducibility of these results and to see if the differences observed here are representative of isolates from the different sequence types.

Lesions were seen in many of the dead fish, but no difference was observed in their prevalence or variety between the two sequence types (Table 1). Hemorrhaging of the fins was the most frequent clinical symptom of VHSV, followed by petechial hemorrhaging, serosanguinous ascites, and cranial hemorrhaging, respectively (Fig. 4). These signs are all consistent with the lesions commonly seen in VHSV-infected fish.

The viral load was measured over time from weekly fin clips of surviving fish as well as pooled organ samples on dead fish. Interestingly, the viral load in the round gobies over the course of the infection trial was not significantly different between the two sequence types (Fig. 5a). Although more experimentation is needed for conclusive data, this supports the observation from the *in vitro* experiments that vcG001 and vcG002 are producing similar amounts of viral RNA, but vcG001 is more efficient at producing infectious viral particles under some conditions. Replicate experiments using smaller dosages of VHSV would aid in further investigating this theory. Likewise in fish that were necropsied because they either died during the trial or because they survived until the end of the trial, pooled organ samples contained no significant difference in viral RNA copies between the two sequence types. Although regulatory testing for VHSV still requires lethal internal organ sampling, recent studies have suggested that non-lethal samples may be equally as effective at detecting

VHSV from fish (Cornwell et al., 2013). In this study, there was good qualitative agreement between a lethal pooled organ sample and a non-lethal fin clip.

The round gobies used for this experiment were wild-caught, and it was thus unknown whether or not some had been previously exposed to VHSV. Because it has been proven that previous exposure to VHSV will increase a fish's immunity to the virus (Hershberger et al., 2011), it is possible that the survivorship was influenced by this unknown factor. Due to the small size of the fish used in this experiment, it was not possible to use serology such as plaque neutralization assays (Millard and Faisal 2012) or competitive ELISA (Millard et al., 2014) to determine prior exposure history. More research comparing the susceptibility of wild-caught versus laboratory-raised round gobies could be performed to understand the probability of this factor.

The differences seen between the two sequence types tested, while small, suggest that not all sequence types of VHSV IVb have the same virulence characteristics. This has potential management consequences because the risk of exposure to different sequence types could be different. Risk-based surveillance has been conducted for VHSV-IVb in the Great Lakes basin for several years (Gustafson et al. 2014). Recently, risk-based management of VHSV has been proposed (Phelps et al. 2014). Knowledge of the different risks posed by different sequence types and their differential spatial distributions such as those described by Thompson et al. (2011) could help inform risk-based models to determine where to place the most efforts on control of spread of the virus and surveillance for virus in wild fish populations.

Conclusions

The results presented in this study provide the first evidence of phenotypic differences between sequence types of VHSV genotype IVb in the Great Lakes. Although the two isolates representing vcG001 and vcG002 tested in this study were similar in many respects, there were significant differences both *in vitro* and *in vivo*. These results have consequences for future management of VHSV in the Great Lakes because different management approaches may be warranted for viruses with different phenotypic characteristics, as well as for the evolution of virulence in RNA viruses.

Acknowledgements

The authors thank Greg Wooster, Ari Fustukjian, and Caroline Laverriere for their technical assistance. S.M.I was supported by a Howard Hughes Medical Institute Fellowship awarded to Kalamazoo College (Kalamazoo, MI). The work described in this report was supported in part by project R/FTD-11 funded under award NA10OAR4170064 from the National Sea Grant College Program of the U.S. Department of Commerce's National Oceanic and Atmospheric Administration, to the Research Foundation of State University of New York on behalf of New York Sea Grant. Mention of trade names does not constitute an endorsement by the U.S. government. The statements, findings, conclusions, views, and recommendations are those of the author(s) and do not necessarily reflect the views of any of those organizations.

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Highlights

Highlights for “*In vivo* and *in vitro* phenotypic differences between Great Lakes VHSV genotype IVb isolates with sequence types vcG001 and vcG002”.

- Phenotypic differences between vcG001 and 002 can be observed *in vitro*.
- Round goby are susceptible to both vcG001 and vcG002.
- At a low exposure dose, round goby are more susceptible to vcG001.

Fig. 1.

Schematic of experimental design for in vivo testing of phenotypic difference between VHSV IVb sequence types vcG001 and vcG002. Controls were exposed to cell culture media without virus.

Fig. 2.

In vitro growth dynamics of VHSV IVb sequence types vcG001 (black line, solid circles) and vcG002 (grey line, open circles). There was no difference in growth kinetics of infectious viral particles between the two sequence types in Chinook salmon embryo cells (CHSE; a), but there was a significant difference in growth kinetics of infectious viral particles between the two sequence types in bluegill fry cells (BF-2; b) and epithelioma papulosum cyprini cells (EPC; c). There was no difference in the amount of viral RNA produced over time in CHSE (d), BF-2 (e), or EPC (f) cell lines. Each point represents the average of three (infectious viral particles) or two (viral RNA) replicates and each line represents a separate growth curve experiment (n=3 per sequence type). Time zero represents when virus was added to the cells.

Fig. 3.

Average survivorship of fish over time infected at three different doses. Survivorship for 10^2 pfu mL⁻¹ (a) was significantly different between vcG001 (solid line) and vcG002 (dashed line). Survivorship differences between sequence types were not significant for 10^3 pfu mL⁻¹ (b) and 10^4 pfu mL⁻¹ (c) treatments. Individuals who died during the trial from a cause other than VHSV or who survived until the end of the trial are represented by a +.

Fig. 4. Lesions in round goby (*Neogobius melanostomus*) infected with VHSV IVb sequence types vcG001 and 002. Fish showed multifocal areas of hemorrhages on fins and body. Scale bars represent 1 cm.

Fig. 5. Difference in viral load in weekly fin samples (a) and at upon death (b) between round goby (*Neogobius melanostomus*) infected with VHSV IVb sequence type vcG001 (closed circles, black line) and vcG001 (open circles, grey line).

Table 1

Frequency of lesions seen in round goby (*Neogobius melanostomus*) that died while infected with VHSV IVb sequence types vcG001 and vcG002. There was no significant difference between the two sequence types in terms of whether or not fish that died during the trial showed any lesions.

Lesion type	vcG001		vcG002	
	Number of fish with lesion (out of 43 fish)	Frequency	Number of fish with lesion (out of 27 fish)	Frequency
Hemorrhaging on fins	22	0.51	11	0.41
Petechial hemorrhaging	8	0.19	7	0.26
Cranial hemorrhaging	7	0.16	4	0.15
Serosanguinous ascites	10	0.23	6	0.22
Hemorrhaging of internal organs	8	0.19	5	0.19
Hemorrhaging on vent	2	0.05	1	0.002
Dermal pallor	0	0	2	0.07